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Hydrogenase-Mediated Activities in Isolated Chloroplasts of *Chlamydomonas reinhardii*[1]

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ABSTRACT

Isolated intact chloroplasts of *Chlamydomonas reinhardii* were found to catalyze photoreduction of CO_2 in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea when adapted under an atmosphere of H_2 demonstrating the association of a hydrogenase and anaerobic adaptation system with these plastids. The specific activity of photoreduction was approximately one third that detected in cells and protoplasts. Photoreduction was found to have a lower osmoticum optimum relative to aerobically maintained chloroplasts (50 millimolar *versus* 120 millimolar mannitol). 3-Phosphoglycerate (3-PGA) stimulated photoreduction up to a peak at 0.25 millimolar beyond which inhibition was observed. In the absence of 3-PGA, inorganic phosphate had no effect on photoreduction but in the presence of 3-PGA, inorganic phosphate also stimulated the reaction. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone inhibited photoreduction but inhibition by the former could be partially overcome by exogenously added ATP. The intact plastid can also catalyze photoevolution of H_2 while lysed chloroplast extracts catalyzed the reduction of methyl viologen by H_2. Both reactions occurred at rates approximately one-third of those found in cells. The oxyhydrogen reaction in the presence or absence of CO_2 was not detected.

The unicellular algae are unique among eukaryotes in their possession of an anaerobically inducible hydrogenase system (5, 7, 11). This enzyme catalyzes the light-driven evolution of H_2 gas, the light-driven consumption of H_2 gas coupled to CO_2 fixation (photoreduction), and the dark consumption of H_2 gas coupled to O_2 and CO_2 reduction (oxyhydrogen reaction) in *Chlamydomonas reinhardii* (4, 5, 9). Either water or carbon substrates can serve as the electron donor to the photosynthetic H_2 evolving system leading to the formation of ATP and eliminating excess reducing potential in the form of H_2 (5). Since photosynthetically produced O_2 can irreversibly inactivate the hydrogenase at very low concentrations (11), the hydrogenase must maintain a very delicate redox balance within the cell for metabolism to continue under anaerobic or microaerobic conditions.

Similar enzyme systems in the blue green algae and bacteria are also closely associated with photosynthetic metabolism, the respiratory electron transport pathway and nitrogenase systems. The energetic association of the hydrogenase with photosynthetic reactions in *C. reinhardii* suggests that in eukaryotic organisms the hydrogenase may be located in the chloroplast, although no direct confirmation of this assumption has been presented. The finding that the hydrogenase of *C. reinhardii* is synthesized on cytoplasmic ribosomes (15) would necessitate that the enzyme be transported across the chloroplast after translation.

The recently developed method for isolating photosynthetically competent chloroplasts from *C. reinhardii* (12) has now made it possible to study the metabolism of these organelles directly. In the present study, we report on the investigation of hydrogenase-catalyzed reactions including photoreduction, oxyhydrogen reaction, H_2 evolution and H_2 uptake in the presence of MV[3] in isolated *Chlamydomonas* chloroplasts. A preliminary report of these finding has been presented (14).

EXPERIMENTAL METHODS

Cell Culture and Synchronization. Cultures of *Chlamydomonas reinhardii* 32/11-b were grown photoautotrophically in an inorganic medium agitated with air containing 1 to 2% CO_2 as previously described (12). When cultures were grown for the production of autolysine *C. reinhardii* 32/11-c was used as the complementary mating type. Cell growth was synchronized to a schedule of 12 h light/12 h dark and was routinely harvested for experimentation midway through the dark phase. At this time cells were in the 4 to 8 sporangial stage.

Preparation of Protoplasts and Chloroplasts. *C. reinhardii* protoplasts were generated by autolysine treatment of sporangial stage cells (16). The extent of protoplast formation could be monitored by their susceptibility to lyse in the presence of 0.1% Triton X-100. For the preparation of intact chloroplasts, protoplasts were lysed by digitonin treatment and intact plastids isolated by Percoll density gradient centrifugation (12). Chloroplasts, at least 90% intact as visualized by phase contrast microscopy, containing less than 3 to 5% protoplasts were used routinely for experiments. In later experiments a chloroplast sample was simultaneously run in the absence of mannitol which served to detect protoplast and cell contamination which were not inhibited by low osmoticum (see Fig. 2 and "Results").

Assay of Hydrogenase Mediated Activities. After isolation, intact chloroplasts were resuspended at a concentration of approximately 1 mg Chl/ml in 20 mM Tricine-NaOH, pH 7.8, 150 mM mannitol, 2 mM EDTA, and 1 mM $MgCl_2$. Chloroplasts equal to 10 to 20 μg Chl (1) were added to serum vials containing this same buffer including 10 μM DCMU and 50 mM mannitol unless otherwise specified. Including all other additions (except

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[3] Abbreviations: MV, methyl viologen; 3-PGA, 3-phosphoglycerate; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; PQ, plastoquinone.

$NaHCO_3$) the volume of the adaptation mixture was 0.9 ml at this stage. The vials were sealed with serum stoppers and deoxygenated by five repetitions of evacuation and flushing with N_2 or H_2 at the reaction temperature of 28°C. The vials were then flushed with the appropriate gas for an additional 20 min. After a total adaptation time of 30 min, 5 μmol of $NaH^{14}CO_3$ (4–10 μCi/mol) were injected through the septa to bring the reaction mixture to a total volume of 1 ml and photoreduction was started by the onset of illumination (100 W/m^2). The oxyhydrogen reaction was assayed in the same manner except that the reaction was conducted in the dark and O_2 gas (final concentration 1% v/v) was added to the atmosphere above the reaction mixture to start the assay. In both reactions, 100 μl aliquots were removed by syringe at several time points, and the reaction terminated by addition of 0.2 ml of 1 N HCl and assayed for incorporation of ^{14}C into nonvolatile material.

Photoevolution of H_2 was measured with a H_2 electrode (Hansatech Limited, Norfolk, England) in the same reaction buffer as described above except that the DCMU was omitted. Chloroplasts or cells were adapted anaerobically with N_2 at 1 mg Chl/ml in 50 mM mannitol buffer, and plastids equal to 20 to 50 μg Chl were injected into the H_2 electrode chamber containing deoxygenated buffer (total volume 1 ml). Best results were obtained if 5 mM sodium dithionite was included in the reaction mixture to scavenge O_2. The temperature controlled chamber was illuminated at 25°C with 100 to 200 W/m^2 ($CuSO_4$-filtered light).

Methyl viologen reduction was performed as described by Erbes *et al.* (3). Chloroplasts were adapted under N_2 at approximately 1 mg Chl/ml in 50 mM mannitol buffer containing DCMU, sonicated and centrifuged anaerobically, and the supernatant assayed for H_2 dependent activity.

RESULTS

Detection of Hydrogenase Activities in *Chlamydomonas* Chloroplasts. Anaerobically adapted *C. reinhardii* chloroplasts showed photoreduction activity equivalent to approximately one-third that detected in the source cells or protoplasts (Fig. 1). The activity was linear for at least 20 min and was strictly dependent on light and an atmosphere containing H_2. It has been shown that hydrogenase activity in *C. reinhardii* cells increases for up to 5 h after the onset of anaerobic conditions 915) which might also be expected to lead to increased photoreductive activity. Adaptation of chloroplasts to anaerobic conditions for a time period longer than 30 min did not enhance photoreduction in chloroplasts further (data not shown).

Preliminary experiments using the hydrogen electrode indicated that these plastids were also capable of photoevolution of H_2 at rates of 5 to 6 μmol/mg Chl·h as compared to 15 to 20 μmol/mg Chl·h detected in whole cells. Similarly, sonically lysed, anaerobically adapted chloroplasts catalyzed the reduction of MV (3) by H_2 (18–50 μmol/mg Chl·h) at approximately 20 to 50% the rate in whole cell preparations. The MV reduction activity was present in the supernatant after centrifugation of the sonicated particles and it was sensitive to trace levels of O_2 as previously reported for whole cell hydrogenase (3). A successful oxyhydrogen reaction in the presence or absence of CO_2 was not accomplished with our chloroplast preparation.

Optimization of Photoreduction Activity in Chloroplasts. Assay of photoreduction activity in *C. reinhardii* chloroplasts under the conditions specified for the measurement of photosynthesis (13) resulted in low and often undetectable activity. Investigation of the concentration dependence for the photoreduction reaction components revealed 50 mM mannitol to be the optimal osmoticum concentration under these conditions (Fig. 2) rather than 120 mM as determined for aerobic photosynthesis (13). The loss of activity in chloroplasts assayed in the absence of mannitol was not seen in cells or protoplasts (data not shown). In all subsequent experiments a chloroplast sample lacking mannitol was also routinely run as a control for cellular and protoplast contamination. If activity in the absence of mannitol was greater than 20 to 25% of that in the presence of 50 mM mannitol then the results were discarded.

A sharp optimum of 0.25 mM was determined for 3-PGA (Fig. 3) in photoreduction which is similar to that determined for photosynthesis in the absence of Pi in these plastids. As expected for chloroplasts containing a Pi/triose-P exchanger in the inner envelope membrane high concentrations of 3-PGA are inhibitory (10). In contrast to aerobic photosynthesis (13) added Pi did not stimulate or inhibit photoreduction significantly in the absence of 3-PGA (Fig. 4). In the presence of 0.25 mM PGA, photoreduction was stimulated up to a maximum at 1 mM Pi with only slight inhibition at higher concentrations again in contrast to the photosynthetic response (13) where inhibition is observed beyond the Pi optimum. Added ribose-5-P stimulated photoreduction up to 10 mM beyond which it inhibited (data not shown).

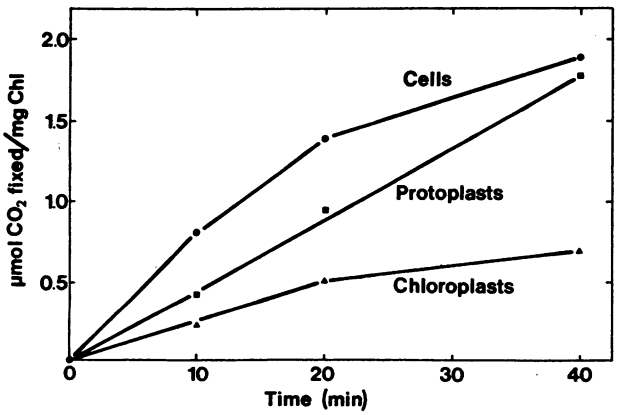

FIG. 1. Measurement of photoreduction in Chl-containing preparations from *Chlamydomonas reinhardii*. Cell (●), protoplasts (■) or chloroplasts (▲) equivalent to 10 to 20 mg Chl were anaerobically adapted with H_2 for approximately 30 min. After adaptation 5 μmol $NaHCO_3$ (4–10 μCi/mol) were injected and the reaction started with the onset of illumination.

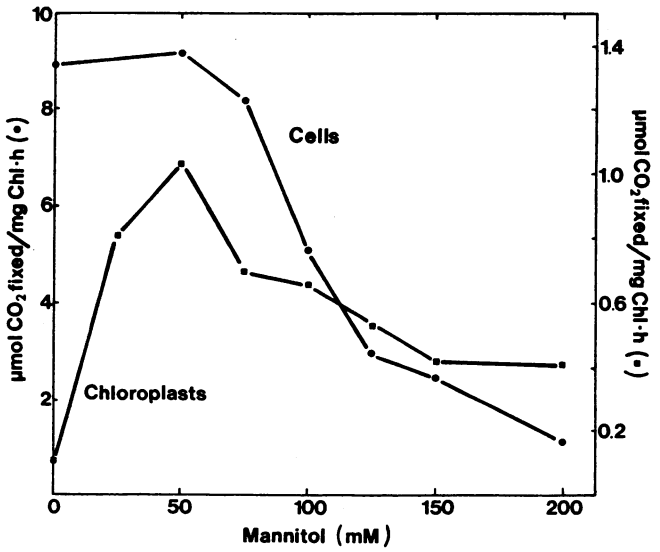

FIG. 2. Optimization of osmoticum concentration for photoreduction in *Chlamydomonas* chloroplasts. Cells (●) or chloroplasts (■) were adapted to H_2 in a reaction buffer containing varying mannitol concentrations. Photoreduction was measured as in Figure 1.

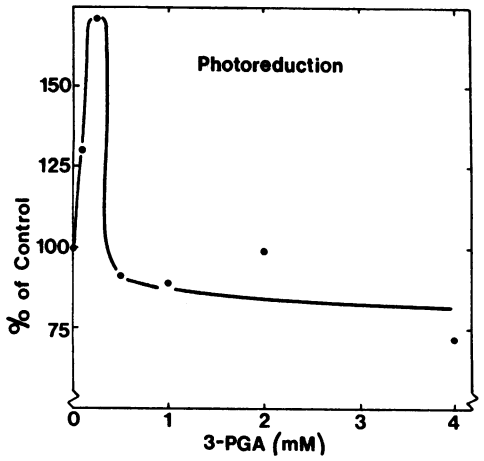

FIG. 3. Stimulation of photoreduction by 3-PGA in chloroplasts. Chloroplasts were anaerobically adapted in a reaction buffer containing varying 3-PGA concentrations and assayed as in Figure 1. A sample containing no mannitol functioned as control for protoplast and cellular contamination. The rate in the absence of 3-PGA was approximately 0.9 μmol CO_2/mg Chl·h.

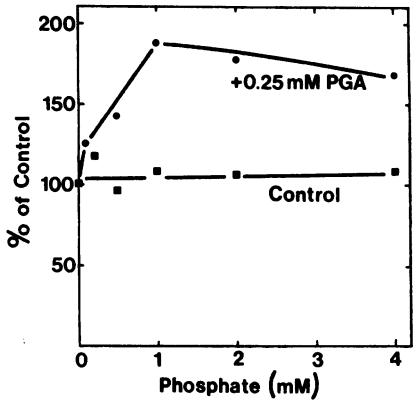

FIG. 4. Sensitivity of photoreduction in chloroplasts to Pi concentration. Chloroplasts were adapted anaerobically in the presence of various concentrations of K-phosphate either in the presence (●) or absence (■) of 0.25 mM 3-PGA. In the absence of Pi the reaction rates were approximately 0.6 μmol CO_2/mg Chl·h (+3-PGA) and 0.4 μmol CO_2/mg Chl·h (no PGA).

Effect of Photosynthetic Inhibitors on Photoreduction. Photoreduction is routinely conducted in the presence of 10 μM DCMU to eliminate water splitting as a source of electrons for CO_2 fixation and O_2 which would inactivate the hydrogenase system. Addition of the quinone analog, DBMIB, to the photoreduction reaction mixture inhibited CO_2 reduction by more than 95% (Fig. 5) indicating the importance of electron transport through the PQ pool for photoreduction to occur. Inhibition of photoreduction by the uncoupler FCCP and partial recovery of activity by the addition of ATP to the reaction medium show that photophosphorylation is also an essential step. Also, under an atmosphere of H_2 in the dark, 10 mM added ATP could drive CO_2 fixation at level approximately equal to that seen in the presence of FCCP and ATP in the light.

DISCUSSION

The ability of the *Chlamydomonas* chloroplast to catalyze reactions involving both uptake and evolution of H_2 clearly demonstrates that the plastid contains a reversibly functional hydrogenase. That the hydrogenase is inducible in aerobically

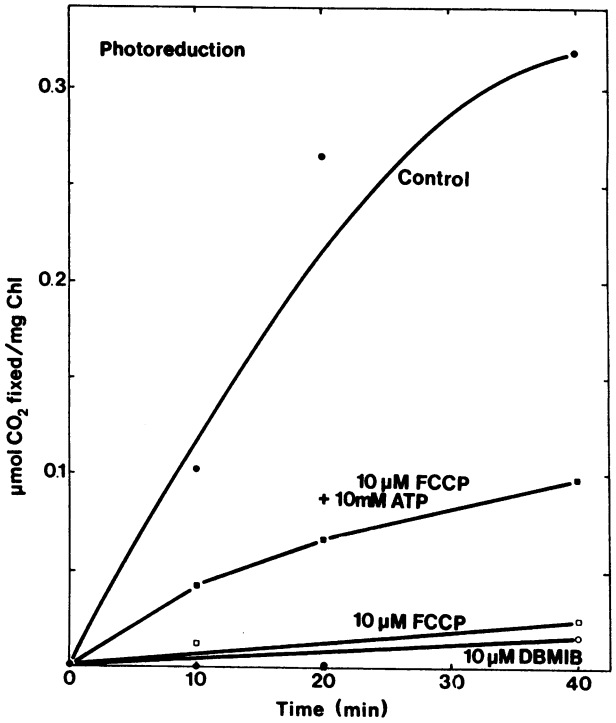

FIG. 5. Sensitivity of chloroplast photoreduction to inhibitors of photosynthesis. Chloroplasts were adapted to H_2 in the presence of the specified additions and assayed as in Figure 1. The control contained 1% (v/v) ethanol which was supplied to the other samples as the solvent for FCCP and DBMIB.

isolated organelles further indicates that the chloroplast also possesses the necessary anaerobic activating system, the nature of which is still obscure. Since the specific activity of the chloroplast hydrogenase (measured as photoreduction) is less than that found in whole cells (Fig. 1) it is not possible to conclude there are no other hydrogenases within the cell. Recent evidence, though, showing the site of synthesis of hydrogenase to be the cytoplasm provides a possible explanation for the differences in specific activities. Chloroplasts would only possess the level of hydrogenase which was present in the cell in the inactive form at the time of isolation while adapted cells would also possess hydrogenase synthesized *de novo* during anaerobic adaptation and assay. Roessler and Lien (15) found that hydrogenase detected during the first 0.5 h of adaptation was due primarily to activation of previously synthesized latent hydrogenase with *de novo* synthesis being expressed later. In our system of synchronously cultured cells with CO_2 as the sole carbon source the effect of *de novo* synthesis may have become important earlier in the time course. Similarly, differences in reaction buffer components may also have had an effect.

Since H_2 photoevolution and MV reduction (H_2 uptake) were also detected in these plastids at similar levels relative to the cell it is possible that one hydrogenase catalyzes all of the H_2-dependent activities and may all be associated with the chloroplast. Examination of the other cellular fractions for hydrogenase activity will be needed to elucidate this point further. *C. reinhardii* chloroplasts were not able to catalyze the oxyhydrogen reaction in the presence or absence of CO_2 under assay conditions where photoreduction was measurable at high rates (data not shown). As mentioned previously externally supplied ATP supported CO_2 reduction in the dark under an atmosphere of H_2. Therefore, we conclude that under an atmosphere of H_2 containing approximately 1% (v/v) O_2 in the dark, the optimal conditions for the oxyhydrogen reaction, the production of ATP is the

limiting step for the reduction of CO_2 in these plastids. It cannot be ruled out, though, that ATP for the oxyhydrogen reaction might be supplied by the mitochondria where another hydrogenase could be located. Investigation of isolated chloroplasts and mitochondria for H_2-dependent ATP synthesis at low O_2 concentrations will be needed to understand the organization of the oxyhydrogen reaction. Our results with MV reduction assay indicate that the chloroplast hydrogenase is extremely sensitive to very low levels of O_2 (data not shown) as reported for the solubilized enzyme (3, 15). The hydrogenase may be protected *in vivo* by stabilizing systems which are not present or do not function in the isolated plastid making the measurement of the oxyhydrogen reaction impossible.

The altered sensitivity of the anaerobically adapted chloroplasts to effectors and substrates of photosynthesis relative to aerobically maintained chloroplasts most likely reflects changes incurred in metabolism and assay, or alterations in chloroplast envelope properties induced by anaerobiosis. Under anaerobic conditions *C. reinhardii* cells are known to metabolize starch to formate, acetate, and ethanol in the dark, and primarily to formate and CO_2 in the light (6), both accompanied by H_2 evolution. Since starch is stored in the chloroplast, this anaerobic metabolism might drastically alter the stromal composition from its aerobic condition. Extensive starch breakdown to triose-P intermediates might account for the lack of inhibition of photoreduction by 1 to 4 mM Pi which ordinarily would deplete the stromal triose-P pool. Alternatively, reductive activation of stromal enzymes under an atmosphere of H_2 could lead to altered metabolite levels in the dark. These imbalances might give rise to the unusual sensitivity of chloroplast photoreduction to exogenous metabolites.

The results of this investigation suggest that reducing equivalents in the form of H_2 can be conserved by the transfer of electrons from hydrogenase probably through the thylakoid NADH-PQ oxidoreductase (2, 8) to the PQ pool. The passage of electrons from PQ through PSI may form a cyclic pathway or lead to NADPH formation and provide the ATP necessary for CO_2 fixation. It is probably the same system by which reducing equivalents are passed from carbon substrates to the electron transport chain resulting in the fermentative dark and photoevolution of H_2 (6, 9). The localization of this system in the chloroplast is presumably not merely an evolutionary artifact since the hydrogenase is synthesized by cytoplasmic ribosomes, and may play an important role in the ecological physiology of these organisms.

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